AD-753 289

JAPANESE ENCEPHALITIS VIRUS REPLICATION: A PROCEDURE FOR THE SELECTIVE ISOLATION AND CHARACTERIZATION OF VIRAL RNA SPECIES

E. Zebovitz, et al

Naval Medical Research Institute

Prepared for:

Bureau of Medicine and Surgery

17 February 1972

UNCLASSIFIED

AD-753289

DOCUMENT CONTROL DATA - R & D

Security classification of title, body of abstract and indexing annotation must be entered when the overall report is classified

1. ORIGINATING ACTIVITY (Corporate author) NAVAL MEDICAL RESEARCH INSTITUTE NATIONAL NAVAL MEDICAL CENTER BETHESDA, MD 20014	2a. REPORT SECURITY CLASSIFICATION UNCLASSIFIED 2b. GROUP

3. REPORT TITLE

JAPANESE ENCEPHALITIS VIRUS REPLICATION: A PROCEDURE FOR THE SELECTIVE ISOLATION AND CHARACTERIZATION OF VIRAL RNA SPECIES

4. DESCRIPTIVE NOTES (Type of report and inclusive dates)
MEDICAL RESEARCH PROGRESS REPORT

5. AUTHOR(S) (First name, middle initial, last name)
E. ZEBOVITZ, J. K. L. LEONG, AND S. C. DOUGHTY

6. REPORT DATE	7a. TOTAL NO. OF PAGES	7b. NO. OF REFS
February 17, 1972	8/11	26

8a. CONTRACT OR GRANT NO.	9a. ORIGINATOR'S REPORT NUMBER(S)
b. PROJECT NO.	MR041.05.01-0006 Report No. 8
c.	9b. OTHER REPORT NO(S) (Any other numbers that may be assigned this report)
d.	

10. DISTRIBUTION STATEMENT

THIS DOCUMENT HAS BEEN APPROVED FOR PUBLIC RELEASE AND SALE: ITS DISTRIBUTION IS UNLIMITED

11. SUPPLEMENTARY NOTES	12. SPONSORING MILITARY ACTIVITY
Reprinted from: Archiv fur die gesame Virusforschung 38, 319-327(1972)	BUREAU OF MEDICINE AND SURGERY (NAVY) DEPARTMENT OF THE NAVY WASHINGTON , D. C. 20390

13. ABSTRACT

The viral RNA species synthesized in a porcine kidney cell lin e, PS(Y-15), by Japanese encephalitis virus (JEV) are described. Virus titers on these cells ranged between 10^6 to 10^7 p.f.u/ml at the end of 2 to 3 days incubation at 35% C. Actinomycin D9AD) mycin D(AD) could not be used to unmask JEV RNA syntehsis since it inhibited virus replication at concentrations necessary to substantially reduce host cell RNA synthesis. Treatment of cells with 1ug AD/ml and removal prior to infection permitted good JEV replication, and at the same time strongly suppressed synthesis of 28 S and 18 S cellular ribosomal RNA. The problem of separatin g viral RNA from non-ribosomal RNA that was still being synthesized by AD pretreatment cells was resolved by the isolation of the cytoplasmic membrane fraction of infected cells. RNA extracted from the membranes of infected Ad pretreated cells and analyzed for sedimentation characteristics on sucrose gradients has four RNA species not found in uninfected cells. They are: (1) 45 S. single stranged RNA believed to be the infectious RNA found in the viron; (2) a 27 S RNA single stranded RNA (3), a 20 S ribonuclease resistant RNA believed to be double stranded and (4) and 8 S RNA species. The RNA species found in JEV infected cells, except for the 8 S form, have been found in group A arboviruses. The procedure described utilizing AD pretreatment of host cells and the separation of the cellular cytoplasmic fraction may well have value for cells and the separation of the cellular cyto-plasmic fraction may well have value for the study of the biosynthetic events involved in the replication of other animal viruses that are inhibited by AD.

DD FORM 1 NOV 65 1473 (PAGE 1)

I

S/N 0102-014-6700

UNCLASSIFIED
Security Classification

KEY WORDS	LINK A		LINK B		LINK C	
	ROLE	WT	ROLE	WT	ROLE	WT
1. Japanese Encephalitis Virus 2. RNA Synthesis						

II

Archiv für die gesamte Virusforschung 38, 319—327 (1972)

Japanese Encephalitis Virus Replication: A Procedure for the Selective Isolation and Characterization of Viral RNA Species[1]

By

E. Zebovitz, J. K. L. Leong[2], and S. C. Doughty

Department of Microbiology, Naval Medical Research Institute, National Naval Medical Center, Bethesda, Maryland, U.S.A.

With 4 Figures

Received February 17, 1972

Summary

The viral RNA species synthesized in a porcine kidney cell line, PS(Y-15), by Japanese encephalitis virus (JEV) are described. Virus titers on these cells ranged between 10^6 to 10^7 p.f.u./ml at the end of 2 to 3 days incubation at 35° C. Actinomycin D (AD) could not be used to unmask JEV RNA synthesis since it inhibited virus replication at concentrations necessary to substantially reduce host cell RNA synthesis. Treatment of cells with 1 μg AD/ml and removal prior to infection permitted good JEV replication, and at the same time strongly suppressed synthesis of 28 S and 18 S cellular ribosomal RNA. The problem of separating viral RNA from non-ribosomal RNA that was still being synthesized by AD pretreated cells was resolved by the isolation of the cytoplasmic membrane fraction of infected cells. RNA extracted from the membranes of infected AD pretreated cells and analyzed for sedimentation characteristics on sucrose gradients has four RNA species not found in uninfected cells. They are: (1) 45 S single stranded RNA believed to be the infectious RNA found in the virion; (2) a 27 S RNA single stranded RNA; (3) a 20 S ribonuclease resistant RNA believed to be double stranded and (4) an 8 S RNA species. The RNA species found in JEV infected cells, except for the 8 S form, have been found in group A arboviruses. The procedure described utilizing AD pretreatment of host cells and the separation of the cellular cytoplasmic fraction may well have value for the study of the biosynthetic events involved in the replication of other animal viruses that are inhibited by AD.

[1] This work was supported by the Bureau of Medicine and Surgery, Department of the Navy, Project MR 041.05.01-0006B3GJ.

The opinions or assertions contained herein are the private ones of the authors and are not to be construed as official or reflecting the views of the Navy Department or the Naval Service at large.

[2] Postdoctoral Research Associate, National Research Council, supported by the Bureau of Medicine and Surgery, Department of the Navy.

E. Zebovitz, J. K. L. Leong, and S. C. Doughty:

1. Introduction

Several ribonucleic acid (RNA) species synthesized in vertebrate cells infected with various group A arboviruses have been isolated and characterized (6, 8, 15, 26). Four species of viral RNA have been separated and classified on the basis of their biological properties, sedimentation properties in sucrose gradients and electrophoretic mobilities in acrylamide gels: a) a 45 S single stranded RNA which is infectious and is found in the mature virion, b) a 27 S single stranded RNA of unknown function, and two ribonuclease resistant double stranded forms, c) a replicative intermediate and d) a replicative form. The precise sequence of synthesis of these RNA products is still unknown.

Similar studies concerned with the various RNA species induced in cells infected with group B arboviruses have been performed but to a lesser extent. Trent *et al.* (23) reported that Saint Louis encephalitis virus induces the synthesis in Vero cells of a 43 S infectious RNA, a 26 S, and 20 S forms as described for group A arboviruses. The 26 S RNA, however, was found to be relatively resistant to ribonuclease. Two viral RNA species, the 45 S and 20 S types, were detected in BHK 21/13 cells infected with type 2 dengue virus (22). In this case the 26 S RNA was present only in trace amount if at all.

The replication of arboviruses is generally considered to be resistant to the action of actinomycin D (AD). In our studies however, we found Japanese encephalitis virus (JEV), a group B arbovirus, to be strongly inhibited by this drug. Standard methods for studying viral RNA synthesis utilizing AD to inhibit host cell RNA synthesis could not be used with JEV.

This report describes a procedure for inhibiting host cell ribosomal RNA synthesis by pretreatment of cells prior to infection with AD and the selective separation of viral RNA from cellular non-ribosomal RNA by the isolation of the cytoplasmic membrane fraction.

2. Materials and Methods

2.1. Cell

A porcine kidney stable cell line designated as PS (Y-15) was selected because it is particularly well suited for plaque formation by JEV (13). Cells were grown on Eagle's minimal essential medium (MEM) supplemented with 10% calf serum and 0.5% lactalbumin hydrolysate on 60 mm tissue culture plates for 3 to 4 days at 37° C in an atmosphere of 5% carbon dioxide in air.

2.2. Virus

JEV strain Yachida of unknown passage history was obtained from Dr. William Allen. Virus seeds consisted of 10% brain suspensions of suckling mice infected intracerebrally.

2.3. Virus Titrations

Confluent monolayers of PS(Y-15) cells were inoculated with 0.1 ml volumes of virus diluted in phosphate-buffered saline (PBS) pH 7.4 containing 0.75% bovine serum albumin. Dilutions were kept in an ice bath until used. Virus was adsorbed to cells for 1 to 2 hours at 35° C and overlayed with 5 ml of a medium composed of MEM containing 1.0% calf serum and 0.8% agarose. Cultures were incubated for 4 days at 35° C in an incubator supplied with 5% carbon dioxide in air. A second overlay consisting of 5 ml of 1:10,000 dilution of neutral red in 1.0% Noble's agar was added.

The plates were returned to the 35° C incubator and the viral plaques counted the following day.

2.4. Infection and AD Pretreatment

Growth medium was removed from the PS(Y-15) monolayers and each culture was treated for 60 minutes with 3 ml of 1 μg/ml AD in PBS. The drug was removed by aspiration and the cells washed twice with PBS. The cultures were infected at a multiplicity of 1 plaque forming unit (p.f.u.) per cell. The virus was allowed to adsorb for 1 hr at 35° C. The cultures were then overlayed with 5 ml Hanks lactalbumin hydrolysate medium (HLH), purchased from Grand Island Biological Co., supplemented with 10% calf serum and incubated at 35° C with 5% carbon dioxide in air. Growth medium was removed on the second day and replaced with 3 ml/plate HLH without calf serum containing 5 μCi ^{3}H-5-uridine/ml. Cultures were reincubated at 35° C for 2 hours. The cells were harvested by scraping into the medium with a rubber policeman and sedimented by centrifugation. The cell pellet was washed once with PBS. The subsequent procedures were performed in an ice bath.

2.5. Preparation of Cytoplasmic Membrane Containing Fraction

The PBS washed cells were suspended in reticulocyte standard buffer (RSB) (0.01 M Tris-HCl; 0.01 M KCl; 1.5×10^{-3} M $MgCl_2$, pH 7.4) and allowed to swell for 10 minutes. The cells were ruptured with 25 strokes in a closely fitting Dounce homogenizer. Large cell debris were pelleted at $600 \times g$ for 5 minutes and discarded. The turbid supernatant was centrifuged (Model L Spinco) at $10{,}000 \times g$ for 10 minutes. The resultant pellet consisted largely of cell membranes, but probably included mitochondria and some ribosomes. This material will be designated as the cytoplasmic membrane containing (CMC) fraction. Approximately 1.5×10^7 to 3.0×10^7 cells were used in the preparation of the CMC fraction.

2.6. Extraction of RNA from CMC Fraction

Immediately after its isolation, the CMC fraction was resuspended in 20 ml of 0.02 M phosphate buffer containing 10^{-3} M EDTA pH 7.4 (5), and extracted twice with 20 ml of cold water-saturated redistilled phenol containing 0.5% sodium dodecyl sulfate. To precipitate the RNA, the aqueous phase obtained after the second phenol extraction was added to 3.5 volumes of cold 95% ethanol containing 0.3% potassium acetate and held at −20° C for a minimum of 2 hours. The sediment was collected by centrifugation, dissolved in 10 ml PBS, precipitated again and stored at −20° C.

2.7. Sucrose Density Gradient Analysis

RNA derived as above from CMC fraction and pelleted from alcohol by centrifugation was dissolved in 2 ml PBS, layered on top of a 28 ml sucrose gradient (15 to 35% sucrose in RSB containing 0.15 M NaCl) and centrifuged in a SW 25.1 rotor at 23,000 r.p.m. in a Model L Spinco for 18 hours. Chick cell RNA was added to the CMC RNA to provide 28 S and 18 S ribosomal RNA species as sedimentation markers. Gradient tubes were pierced at the bottom with an 18 gauge needle; 10 drop fractions were collected and diluted to 1.0 ml volumes with PBS. The absorbance at 260 nm of each fraction was determined to localize the position of the chick cell ribosomal RNA peaks.

Acid insoluble radioactivity was measured by precipitating the fractions in the presence of 500 μg yeast RNA as carrier with 10% trichloroacetic acid (TCA). To determine the position of the ribonuclease resistant double stranded viral RNA, each fraction was divided into two 0.5 ml aliquots. One aliquot was treated with 1 μg pancreatic ribonuclease for 30 minutes at 37° C, and the acid insoluble RNA precipitated as described above. The second aliquot was processed without ribonuclease treatment.

The TCA insoluble precipitates were collected on membrane filters of 3.0 μm pore size. The amount of radioactivity trapped on dried filters placed in 10 ml of a liquid scintillator solution [4 gm Omnifluor (New England Nuclear) in one liter toluene] was determined with a Beckman LS-250 liquid scintillation spectrometer.

Sedimentation coefficients were calculated by the method of Martis and Ames (17) using chick cell 28 S and 18 S ribosomal RNA as sedimentation markers.

3. Results

3.1. The Effect of AD on JEV Replication

PS(Y-15) cells support good growth of JEV and titers ranging between 10^6 and 10^7 p.f.u./ml are reached at the end of 2 to 3 days incubation at 35° C.

AD has a pronounced inhibitory effect upon the replication of JEV (Fig. 1). The inhibition is dose-dependent and virus growth is inhibited more than 90% in the presence of 1 μg AD/ml. Several JEV strains, available in our laboratory, Nakayama, Peking and a Taiwan strain 143 were also sensitive to AD when grown

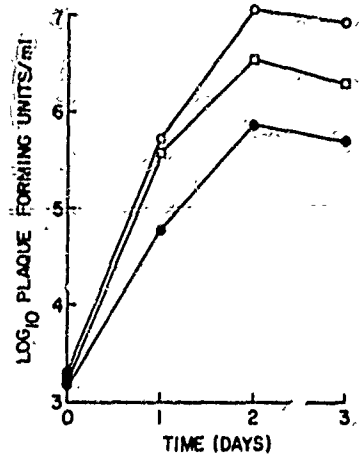

Fig. 1. The effect of AD upon JEV replication on PS(Y-15) cells. 5 ml HLH medium without calf serum containing the desired concentration of AD was added to each culture after infection. Samples of pooled medium from duplicate cultures were taken at daily intervals and plaque assays performed as described in Materials and Methods. o——o 0 μg/ml, □——□ 0.1 μg/ml, ●——● 1.0 μg/ml

on PS(Y-15) cells. These results indicate that AD can not be used to unmask JEV RNA synthesis since it inhibits virus replication at levels necessary to sufficiently reduce host cell RNA synthesis. Another method is therefore required to selectively inhibit host cell RNA synthesis.

3.2. Pretreatment of Cells with AD

It was shown that treatment of normal cells with AD for a short period followed by its removal resulted in a markedly altered pattern of cellular RNA synthesis. Ribosomal RNA synthesis is particularly sensitive to AD and remains inhibited even after the drug is removed (20). Reduction of the amount of ribosomal RNA made in infected cells is essential for the detection of viral RNA since the RNA species made in infected cells have sedimentation values very similar to the 28 S and 18 S ribosomal RNA species.

To test the effect of pretreatment of PS(Y-15) cells with AD upon JEV replication and cellular ribosomal RNA synthesis, the cells were incubated with increasing levels of AD for 60 minutes, the drug removed and infected with JEV. Viral growth after pretreatment with several AD concentrations is shown in Figure 2. High concentrations of AD from 1 to 10 μg/ml did not adversely affect virus

growth and titers obtained at 2 days equalled that of the untreated control cultures.

The sedimentation characteristics of RNA obtained from uninfected PS(Y-15) cells treated with 1 μg/ml and 5 μg/ml AD in the manner described above and

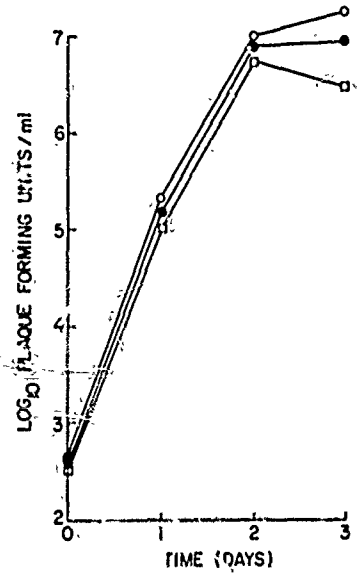

Fig. 2. The effect of pretreatment of PS(Y-15) cells with AD upon growth of JEV. Cells were treated with diluted concentration of AD in 3 ml PBS for 60 minutes. The medium was removed and cells washed twice with PBS before infection. Plates were overlayed with 5/ml HLH medium with 10% calf serum. Samples of pooled growth medium from duplicate plates were taken daily. Virus titers were determined by plaque assay as described in Materials and Methods.
o——o 0 μg AD/ml, •——• 1.0 μg AD/ml, □——□ 10 μg AD/ml

incubated with radioactive uridine for 2 days are illustrated in Figure 3. The short AD treatment with 1 μg/ml AD resulted in a partial inhibition of 28 S and 18 S ribosomal RNA synthesis and an increase in the amount of RNA that sediments in the 5 to 10 S region. Five μg AD/ml almost completely inhibited ribosomal RNA synthesis, but the amount of slowly sedimenting RNA was not increased above that obtained with 1 μg AD. The presence of appreciable amounts of non-ribosomal RNA, however, required an additional procedure for separation of host and viral RNA products.

3.3. Isolation of Viral RNA from CMC Fraction

Membranes of the endoplasmic reticulum of the host cell have been implicated in RNA virus replication (1, 9, 11). Viral protein, RNA, and RNA polymerase were found to be associated with this cell fraction. JEV, like other arboviruses, develops on the cytoplasmic membranes (18). This association of viral RNA with the cytoplasmic membranes seemed to offer the means of separating viral RNA from the cellular RNA that is not inhibited by short treatment with AD.

RNA extracted from the CMC fraction of normal and infected cells contained approximately 10 and 25%, respectively, of the radioactivity originally incorporat-

ed into the intact cells. These data indicate that the procedure of isolating the CMC fraction prior to RNA extraction removes a substantial amount of cellular RNA.

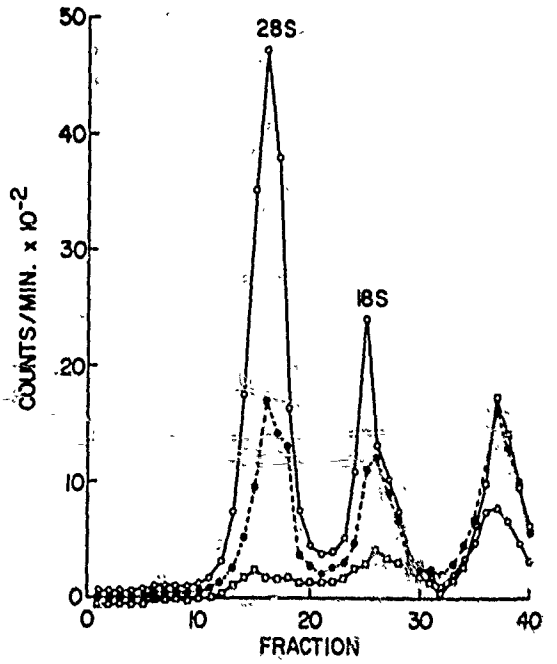

Fig. 3. The effect of pretreatment of PS(Y-15) cells with AD upon ribosomal RNA synthesis. Cells were treated with AD for 60 minutes. Residual AD was removed by two washes with PBS. The cells were overlayed with 3 ml HLH medium with 1 μC/ml ^{3}H-uridine and incubated at 35 °C for 2 days. Ribosomal RNA was extracted from intact cells with cold phenol by the procedure described for CMC fraction in Materials and Methods.
o——o untreated control, ●– –● 1.0 μg/ml, ▪— —▪ 5.0 μg/ml

The sedimentation patterns of RNA extracted from control and infected CMC fraction from AD pretreated cells were markedly different (Fig. 4 A). The RNA in each case was derived from approximately the same number of cells, yet the radioactivity recovered from the control cells was much less than that of infected cells and no major peaks were observed. The amount of host cell RNA of low sedimentation coefficient, shown in Figure 3, was greatly reduced by this procedure.

Two major and two minor species of viral RNA are found associated with the CMC fraction. The most rapidly sedimenting species, 45 S, has approximately the same sedimentation coefficient as that determined by Igarashi *et al.* for the infectious RNA of JEV (12). The second major peak sediments at 20 S and is resistant to ribonuclease (Fig. 4 B) and probably represents the double stranded viral RNA species. The two small but distinct peaks consist of a 27 S RNA, which corresponds to the one isolated in group A arboviruses in much larger amounts, and an 8 S RNA which is possibly of viral origin but whose significance in JEV replication is not yet known.

The possibility that the separation of the CMC fraction would be sufficient to selectively isolate JEV RNA products from cellular RNA without first treating

the cells with AD was tested. Sedimentation pattern of RNA extracted from the CMC fraction of untreated infected cells consisted primarily of the two cellular ribosomal RNA species. Viral RNA peaks were completely masked by those of the cellular RNA. AD treatment of the cells prior to infection is an essential step to suppress ribosomal RNA synthesis.

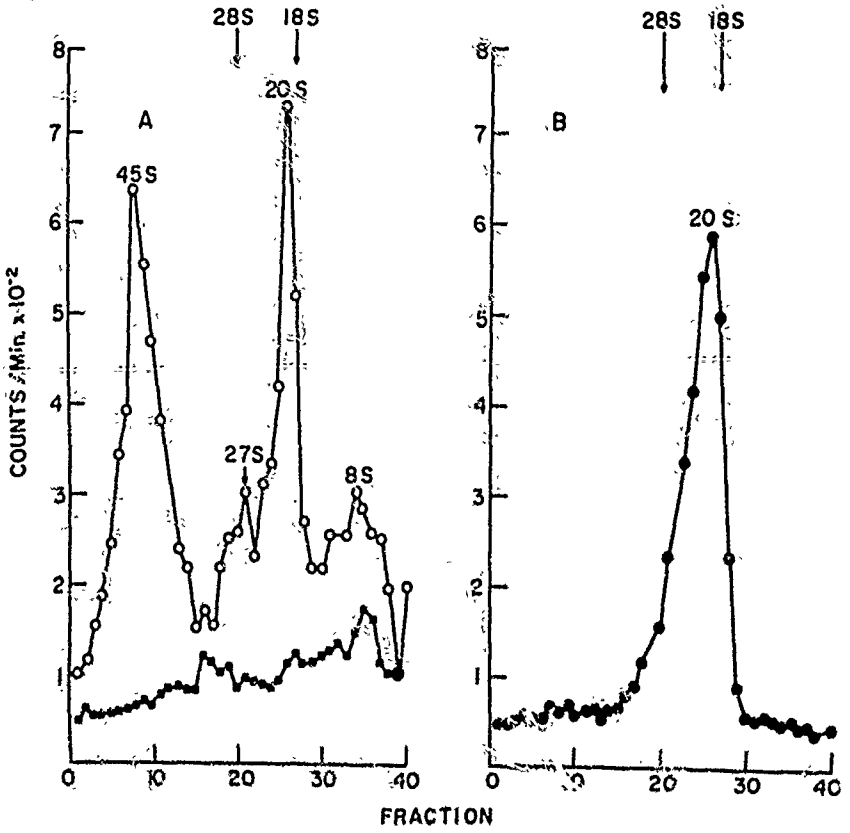

Fig. 4. Sedimentation profile of RNA extracted from CMC fraction derived from PS(Y-15) cells infected with JEV. JEV infected and control uninfected cells pretreated with 1 μg/ml AD were incubated at 35 °C for 2 days. Cells were pulse labeled with 5 μCi ^{3}H-5-uridine for 2 hours. CMC fraction RNA isolated and analyzed as described in Materials and Methods.
A. Viral RNA (o- - -o) and control cell RNA (■ — ■). B. Ribonuclease resistant viral RNA (● · · ●)

4. Discussion

The RNA species synthesized in JEV infected PS(Y-15) cells are similar to those reported for group A arboviruses (6, 8, 15, 20), except that the amount of 27 S RNA in relation to 45 S and 20 S RNA's that are in this case associated with the CMC fraction appeared to be much smaller. The low level of 27 S RNA recovered may be the result of the procedure used for the isolation of the cytoplasmic membranes. Destruction of the 27 S RNA by the nucleases present in the cell extracts is possibly not the complete explanation, because RNA retained on the cell membranes is believed to be resistant to nuclease action (1, 9). Also the 45 S RNA, a single stranded RNA, appears to be retained intact. However, it is

possible that the 27 S RNA is loosely bound to the membrane and some is released from it during the isolation procedure. If this is the case this RNA fraction would be discarded or exposed to nuclease digestion. Attempts to detect this RNA in the cell extract after removal of the membrane fraction have failed. Finally, it is possible that the low level of 27 S RNA is a property of JEV. A similar observation was made for type 2 dengue virus by Stollar *et al.* (22) who reported that 26 S RNA was found in infected cells in very small amounts or not at all.

Study of RNA synthesis by JEV was complicated by the sensitivity of this virus to AD. JEV is not unique in this respect since a number of RNA viruses are known to be inhibited by AD perhaps for different reasons. Under certain conditions the growth of some strains of poliovirus (10, 19), reovirus (16), mumps virus (7), fowl plague virus (2), lymphocytic choriomeningitis virus (4), and the RNA tumor viruses (3, 24) is inhibited by AD.

The effect of AD has been tested with only a few members of group B arboviruses. Levels in the range of 0.01 to 0.03 μg/ml AD have a slight stimulating effect upon dengue (21) but inhibit virus growth at concentrations in excess of 0.1 μg/ml. An enhancement effect by low AD concentrations has also been reported for JEV (25). The increased virus yield in both cases was attributed to the inhibition by AD of interferon synthesis. St. Louis encephalitis virus is reported to replicate normally in BHK-21/13 cells in the presence of AD at 0.5 μg/ml and the synthesis of viral RNA was studied under conditions in which cellular RNA synthesis was inhibited (23). In our system JEV was inhibited by AD at this concentration.

The mechanism of inhibition of JEV by AD is not known. The possibility that a subtle cellular function or virus directed activity may be involved is now being investigated.

The procedure described for the selective isolation of JEV RNA utilizing AD pretreatment of host cells and the separation of the cellular cytoplasmic fraction may well have value for the study of the biosynthetic events involved in the replication of other animal viruses that are inhibited by AD.

Acknowledgments

The authors deeply appreciate the excellent technical assistance of Mr. Richard Grays and Mr. Rodney T. Miller.

References

1. Baltimore, D., H. J. Eggers, R. M. Franklin, and I. Tamm: Poliovirus-induced RNA polymerase and effects of virus specific inhibitors on its production. Proc. Nat. Acad. Sci. (Wash.) **49**, 843–849 (1963).
2. Barry, R. D.: The effects of actinomycin D and ultraviolet irradiation on the production of fowl plague virus. Virology **24**, 563–569 (1964).
3. Bases, R. E., and A. S. King: Inhibition of Rauscher murine leukemia virus growth in vitro by actinomycin D. Virology **32**, 175–183 (1967).
4. Buck, L. L., and C. J. Pfau: Inhibition of lymphocytic choriomeningitis virus replication by actinomycin D and 8-azauridine. Virology **37**, 698–701 (1969).
5. Colon, J. L., and J. B. Dodge: Factors affecting plaque formation by the infectious ribonucleic acid of the equine encephalitis viruses. J. infect. Dis. **114**, 61–68 (1964).
6. Donos, P., and P. Faulkner: Properties of 42 S and 26 S Sindbis viral ribonucleic acid species. J. Virol. **4**, 429–438 (1969).

7. East, J. L., and D. W. Kingsbury: Mumps virus replication in chick embryo lung cells: Properties of RNA in virions and infected cells. J. Virol. 8, 161—173 (1971).
8. Friedman, R. M., H. B. Levy, and W. B. Carter: Replication of Semliki Forest virus: Three forms of viral RNA produced during infection. Proc. nat. Acad. Sci. (Wash.) 56, 440—446 (1966).
9. Girard, M., D. Baltimore, and J. E. Darnell: The poliovirus replication complex: Site for synthesis of poliovirus RNA. J. molec. Biol. 24, 59—74 (1967).
10. Grado, C., S. Fischer, and G. Contreras: The inhibition by actinomycin D of poliovirus multiplication in Hep 2 cells. Virology 27, 623—625 (1965).
11. Grimley, P. M., I. K. Berezesky, and R. M. Friedman: Cytoplasmic structures associated with an arbovirus infection: Loci of viral ribonucleic acid synthesis. J. Virol. 2, 1326—1338 (1968).
12. Igarashi, A., F. Fukunaga, and K. Fukai: Sedimentation characteristics of Japanese encephalitis virus RNA. Biken J. 7, 111—119 (1964).
13. Inoue, Y. K., and M. A. Yamada: Clonal line of porcine kidney stable cells for assay of Japanese encephalitis virus. J. Bact. 87, 1239—1240 (1964).
14. Leventow, L., and J. M. Bishop: Comparative aspects of poliovirus replication. J. cell. comp. Physiol. 76, 265—272 (1970).
15. Levin, J. G., and R. M. Friedman: Analysis of arbovirus ribonucleic acid forms by polyacrylamide gel electrophoresis. J. Virol. 7, 504—514 (1971).
16. Loh, P. C., and M. Soergel: Growth characteristics of reovirus type 2: Actinomycin D and synthesis of viral RNA. Proc. nat. Acad. Sci. (Wash.) 54, 857—863 (1965).
17. Martin, R. G., and B. N. Ames: A method for determining the sedimentation behavior of enzymes: Application to protein mixtures. J. biol. Chem. 236, 1372—1379 (1961).
18. Ota, Z.: Electron microscope study of the development of Japanese B encephalitis virus in porcine kidney stable (PS) cells. Virology 25, 372—378 (1965).
19. Schaffer, F. L., and M. Gordon: Differential inhibitory effects of actinomycin D among strains of poliovirus. J. Bact. 91, 2309—2316 (1966).
20. Schluederberg, A., R. C. Handel, and S. Chavanich: Actinomycin D: Renewed RNA synthesis after removal from mammalian cells. Science 172, 577—579 (1971).
21. Stollar, V., T. M. Stevens, and R. W. Schlesinger: Studies on the nature of dengue viruses. II. Characterization of viral RNA and effects of inhibitors of RNA synthesis. Virology 30, 303—312 (1966).
22. Stollar, V., R. W. Schlesinger, and T. M. Stevens: Studies on the nature of dengue viruses. III. RNA synthesis in cells infected with type 2 dengue viruses. Virology 33, 650—658 (1967).
23. Trent, D. W., C. C. Swensen, and A. A. Qureshi: Synthesis of Saint Louis encephalitis virus ribonucleic acid in BHK-21/13 cells. J. Virol. 3, 385—394 (1969).
24. Vigier, P., and A. Gaillard: Effects of actinomycin D and of mitomycin C on the development of Rous sarcoma virus. Virology 23, 511—519 (1964).
25. Yamazaki, S.: Effect of endogenous interferon and actinomycin D on growth of Japanese encephalitis virus in chick embryo cells. Jap. J. Microbiol. 12, 171—178 (1968).
26. Zebovitz, E., and A. Brown: Altered viral ribonucleic acid synthesis by a temperature-sensitive mutant of eastern equine encephalitis virus. J. molec. Biol. 50, 185—198 (1970).

Authors' address: Dr. Eugene Zebovitz, Department of Microbiology, Naval Medical Research Institute, Bethesda, MD 20014, U.S.A.

Printed in Austria